-1-

Date;_____ .   Express Mail Label No._____

Inventor(s):   Barbara A. Gilchrest, Mina Yaar, Mark Eller
Attorney's Docket No.:   BU94-15A2

METHODS OF INDUCING HAIR GROWTH AND COLORATION

RELATED APPLICATION(S)

This application is a Continuation-in-Part of U.S. Serial No.: 08/793,683 filed August 30, 1995, which is the
5   U.S. National Phase of PCT/US95/10971 filed 8/30/95 which is a Continuation of U.S. Serial No. 08/298,941, filed 8/31/94, the entire teachings of which are incorporated herein by reference.

BACKGROUND OF THE INVENTION

10   Normal hair follicles cycle between a growth stage (anagen), a degenerative stage (catagen), and a resting stage (telogen). The scalp hairs have a relatively long life cycle: the anagen stage ranges from two to five years, the catagen stage ranges from a few days to a few
15   weeks, and the telogen stage is approximately three months (Fitzpatrick, T.B., et al., eds., DERMATOLOGY IN GENERAL MEDICINE (Vol. I), McGraw-Hill, Inc., 1993, pp. 290-291; Sperling, L.C., J. Amer. Acad. Dermatology (v. 25, No. 1, Part 1), pp. 1-17 (1991)). Shorter hairs found elsewhere
20   on the body have corresponding shorter anagen duration. The morphology of the hair and the hair follicle changes dramatically over the course of the life cycle of the hair.

During anagen, the hair follicle is highly active metabolically (Sperling, L.C., J. Amer. Acad. Dermatology

(v. 25, No. 1, Part 1), p. 4 (1991)). The follicle comprises a follicular (dermal) papilla at the base of the follicle; epidermal matrix cells surrounding the follicular papilla and forming the base of a hair shaft; and the hair

5 shaft that extends upwards from the papilla through the hair canal (Fitzpatrick, T.B., et al., eds., DERMATOLOGY IN GENERAL MEDICINE (Vol. I), McGraw-Hill, Inc., 1993). The matrix cells are the actively growing portion of the hair (Sperling, L.C., J. Amer. Acad. Dermatology (v. 25, No. 1,

10 Part 1), p.6 (1991)). At catagen, the matrix cells retract from the papilla, and other degenerative changes occur (Sperling, L.C., J. Amer. Acad. Dermatology (v. 25, No. 1, Part 1), pp. 13-14 (1991)). A column of epithelial cells pushes the keratinized proximal shaft of the hair upwards

15 (Sperling, L.C., J. Amer. Acad. Dermatology (v. 25, No. 1, Part 1), p. 3 (1991)), and cell death occurs within the follicle (Fitzpatrick, T.B., et al., eds., DERMATOLOGY IN GENERAL MEDICINE (Vol. I), McGraw-Hill, Inc., 1993, p. 291).

20 When the hair follicle reaches the telogen stage, the existing hair has a club-shaped proximal end, and a small bud (a remnant of the epithelial column that is found in catagen) at the base of the follicle (Sperling, L.C., J. Amer. Acad. Dermatology (v. 25, No. 1, Part 1), p. 3

25 (1991)). A telogen hair will not grow further (Fitzpatrick, T.B., et al., eds., DERMATOLOGY IN GENERAL MEDICINE (Vol. I), McGraw-Hill, Inc., 1993, p. 291).

The pigmentary system that colors hair involves melanocytes located in the matrix area of the follicle,

30 above the follicular papilla (Fitzpatrick, T.B., et al., eds., DERMATOLOGY IN GENERAL MEDICINE (Vol. I), McGraw-Hill, Inc., 1993, p. 292). Melanin pigments produced by the melanocytes flow along dendritic processes (Fitzpatrick, T.B., et al., eds., DERMATOLOGY IN GENERAL

MEDICINE (Vol. I), McGraw-Hill, Inc., 1993, p. 292). The
dendritic processes are phagocytized by the differentiating
matrix cells that become part of the hair shaft;
degradation of the phagocytosed material results in release
5 of melanin granules into the cytoplasm (Fitzpatrick, T.B.,
et al., eds., DERMATOLOGY IN GENERAL MEDICINE (Vol. I),
McGraw-Hill, Inc., 1993, p. 671), thus pigmenting the hair.

     Alterations in normal hair pigmentation or growth may
be caused by age, physiologic disease conditions, or injury
10 especially, for example, exposure to ultraviolet-
irradiation. The "graying" of hair, both normal (age-
associated) or abnormal, is known as canities. Graying
results from a progressive decrease in pigment present in
the hair shaft, caused by loss of melanocytes (Fitzpatrick,
15 T.B., et al., eds., DERMATOLOGY IN GENERAL MEDICINE (Vol.
I), McGraw-Hill, Inc., 1993, p. 671; Gilchrest, B.A., SKIN
AND AGING PROCESSES, CRC Press, 1984, p. 19). A decrease
in the density of hair follicles is also associated with
advancing age (Gilchrest, B.A., SKIN AND AGING PROCESSES,
20 CRC Press, 1984, p. 20).

     To date, the mechanism of melanocyte and keratinocyte
injury, for example, from ultraviolet exposure or the aging
process, has not been determined. Thus, little is known or
available regarding a mechanism to manipulate the injury
25 process to prevent cell death and thus prevent premature
baldness or graying of hair or, conversely, to promote
cell death and thus, unwanted hair growth.

SUMMARY OF THE INVENTION
     The present invention is based on Applicants'
30 discovery that basal layer epidermal melanocytes and
keratinocytes undergo characteristic programmed cell death
in response to injury. In particular, Applicants have
shown that epidermal melanocytes and keratinocytes undergo

programmed cell death, or apoptosis, and that apoptosis in these cells is mediated by the p75 nerve growth factor receptor/nerve growth factor pathway (p75 NGF-R/NGF), resulting in upregulation of Bcl-2 protein. As a result of

5 Applicants' discovery, methods are herein provided to control, or manipulate, melanocyte and keratinocyte cell death by altering the effects of apoptosis. For example, apoptosis can be inhibited using methods described herein, resulting in hair growth and coloration. Conversely,

10 apoptosis can be promoted by methods described herein, resulting in hair loss or depigmentation.

Keratinocytes and melanocytes of the basal layer of the epidermis express the high affinity (trk E and trk) and the low affinity (p75) NGF receptors (NGF-R). NGF, known

15 to be produced by keratinocytes, protects cells from death when it binds to NGF receptors. In cells, this NGF effect is mediated in part by induction of the protective protein Bcl-2. Interestingly, basal epidermal keratinocytes and melanocytes express Bcl-2 protein. Specifically, as

20 described herein, it has now been demonstrated that melanocytes expressing the p75 NGF-R can be rescued from apoptotic cell death by the occupation of the p75 NGF-R with NGF or a substance or pseudo-ligand capable of binding to the p75 NGF-R, which initiates the expression of the

25 Bcl-2 protein.

Also as described herein, Applicants have now demonstrated that normal anagen hair follicles strongly express the p75 NGF-R and that p75 NGF-R expression is significantly reduced and limited to a few basal

30 keratinocytes in telogen hair follicles.

As a result of these discoveries, methods are now available for inhibiting the process of apoptosis, or programmed cell death, in basal layer epidermal and follicular keratinocytes and melanocytes in vertebrates,

specifically in humans. Thus, as a result of inhibition of apoptosis, the present invention relates to methods of inducing hair growth and coloration, and delaying hair loss and graying, as well as methods of inducing skin coloration

5   in vertebrates. In addition, the present invention relates to methods of treating alopecia areata and baldness, as well as methods of preventing unwanted hair growth.

In one embodiment of the present invention, the invention relates to a method of preventing melanocyte loss

10 after injury by inhibiting apoptosis in epidermal melanocytes. As described herein, Applicants have now demonstrated that p75 NGF-R mediated apoptosis is responsible for melanocyte loss after injury, for example, due to ultraviolet irradiation or aging. Specifically,

15 Applicants have shown that an unoccupied p75 NGF-R (i.e., a p75 NGF-R which is not bound to ligand such as NGF) induces apoptotic cell death in melanocytes. Thus, ensuring that the p75 NGF-R is occupied by ligand (or pseudo-ligand such as a peptide containing the sequence lysine-glycine-

20 alanine), inhibits the p75 NGF-R induced apoptotic pathway of cell death, resulting in the continued growth/ proliferation, pigment production and pigment transfer to keratinocyte by epidermal melanocytes. Alternatively, epidermal melanocyte cell loss can be prevented by

25 upregulating the expression of Bcl-2 protein in epidermal melanocytes, or by downregulating the expression of the p75 NGF-R in the melanocytes.

In another embodiment of the invention, the invention relates to a method of inducing hair growth in a vertebrate

30 by upregulation of the expression of the p75 NGF-R on keratinocytes in a vertebrate, such as humans, by introducing into epidermal keratinocytes a nucleotide sequence encoding the p75 NGF-R. The p75 NGF-R gene product is expressed on the surface of the keratinocytes,

and becomes available to bind to its naturally occurring ligand, NGF, or to another substance that mimics the binding activity of NGF (i.e., a pseudo-ligand). The p75 NGF-R binds its ligand, or pseudo-ligand, resulting in the

5 expression of the protein, Bcl-2, which protects the keratinocyte from apoptosis.

Alternatively, the upregulation of the expression of the p75 NGF-R can be accomplished by introducing into the keratinocyte a substance, such as a transcription activator

10 protein, which initiates the transcription of the p75 NGF-R gene.

Hair growth can also be induced or prolonged by the upregulation of the expression of the Bcl-2 protein in the keratinocytes, either by the introduction of a nucleotide

15 sequence encoding the Bcl-2 protein or by the introduction of a substance that initiates transcription of the gene encoding the Bcl-2 protein.

In another embodiment of the present invention, the invention relates to a method of inducing hair color in a

20 vertebrate, such as a human, by inhibiting p75 NGF-R mediated apoptosis of epidermal melanocytes. As described above, an unoccupied p75 NGF-R induces apoptosis in epidermal melanocytes. Ensuring that the p75 NGF-R is occupied by ligand, or pseudo-ligand upregulating the

25 expression of Bcl-2 protein, or downregulating the expression of p75 NGF-R inhibits apoptosis in epidermal melanocytes.

In another embodiment of the present invention, the invention relates to a method of inducing skin color in a

30 vertebrate, particularly a human, by inhibiting p75 NGF-R mediated apoptosis of epidermal melanocytes. As described above, an unoccupied p75 NGF-R induces apoptosis in epidermal melanocytes. Ensuring that the p75 NGF-R is occupied by ligand, or pseudo-ligand upregulating the

expression of Bcl-2 protein, or downregulating the expression of p75 NGF-R inhibits apoptosis in epidermal melanocytes. Applicants have shown that peptides containing the sequence lysine-glycine-alanine (KGA)

5 specifically bind p75NGF-R and inhibit p75NGF-R mediated apoptosis of melanocytes.

Conversely, apoptosis can be promoted in melanocytes and keratinocytes in humans, resulting in cell death. For example, cell death may be desirable to prevent unwanted

10 hair growth (e.g., on women's faces or forearms). This can be accomplished, for example, by blocking nerve growth factor from binding to p75 NGF-R, thereby decreasing, or completely inhibiting production of Bcl-2 protein. Thus, apoptotic cell death would be promoted.

15 Another embodiment of the present invention relates to a method of identifying a substance capable of inhibiting apoptosis in melanocytes or keratinocytes by determining the effect the substance has on p75 nerve growth factor receptor.

20 Alternatively, the method of identifying a substance capable of inhibiting apoptosis in melanocytes or keratinocytes can be accomplished by determining the effect the substance has on Bcl-2 protein expression.

Thus, as a result of Applicants' discovery of the role

25 of p75 NGF-R induced apoptosis in epidermal melanocytes, methods are now available to inhibit apoptotic cell death in epidermal and follicular melanocytes, as well as epidermal and follicular keratinocytes, including methods of inducing or prolonging hair growth, hair coloration and

30 skin coloration.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1A is a photomicrograph depicting the effect of UV irradiation with 10 mJ/cm^2 on melanocytes.

Figure 1B is a photomicrograph depicting the effect of sham irradiation on melanocytes.

Figure 1C is a photomicrograph depicting the effect of UV irradiation with 10 mJ/cm^2 on MM4 cells.

5 Figure 1D is a photomicrograph depicting the effect of sham irradiation on MM4 cells.

Figure 1E is a photograph of an agarose gel stained with ethidium bromide depicting the effect of UV irradiation, of MM4 cells on DNA fragmentation.

10 Figure 1F is a photomicrograph depicting the effect of UV irradiation of melanocytes on fragmentation of nuclear chromatin.

Figure 1G is a photomicrograph depicting the effect of UV irradiation of melanocytes on homogenization of nuclear

15 chromatin.

Figure 1H is a bar graph depicting the percentage of propidium iodide-positive melanocytes after sham irradiation, UV irradiation with 10 mJ/cm^2 or UV irradiation with 25 mJ/cm^2.

20 Figure 2A is bar graph depicting cell yields of melanocytes after 3 daily UV irradiations of 0, 5, 10 and 25 mJ/cm^2.

Figure 2B is a bar graph depicting cell yields of MM4 after a single UV irradiation of 10 mJ/cm^2 and

25 supplementation with 50 ng/ml NGF or diluent alone.

Figure 2C is a bar graph depicting cell yields of MM4 after sham irradiation and supplementation with 50 ng/ml NGF or diluent alone.

Figure 2D is a bar graph depicting cell yields of MM4

30 after a single UV irradiation of 10 mJ/cm^2 and supplementation with 50 ng/ml bFGF or diluent alone.

Figure 2E is a bar graph depicting cell yields of MM4 after sham irradiation and supplementation with 50 ng/ml bFGF or diluent alone.

Figure 2F is a photomicrograph depicting melanocyte cell morphology after UV irradiation daily for three days with 10 mJ/cm^2 and supplemented with diluent alone.

Figure 2G is a photomicrograph depicting MM4 cell

5   morphology after UV irradiation once with 10 mJ/cm^2 and supplemented with diluent alone.

Figure 2H is a photomicrograph depicting melanocyte cell morphology after UV irradiation daily for three days with 10 mJ/cm^2 and supplemented with 50 ng/ml NGF.

10   Figure 2I is a photomicrograph depicting MM4 cell morphology after UV irradiation once with 10 mJ/cm^2 and supplemented with 50 ng/ml NGF.

Figure 3A is a photograph of an agarose gel stained with ethidium bromide depicting the effect of UV

15   irradiation of MM4 cells supplemented with NGF on DNA fragmentation.

Figure 3B is a bar graph depicting the percentage of propidium iodide-positive melanocytes after sham irradiation or UV irradiation with 10 mJ/cm and then

20   treatment with 50 ng/ml NGF or diluent alone.

Figure 4A is a graphic representation depicting the effect of NGF on Bcl-2 expression in MM4 cells UV-irradiated with 10 mJ/cm^2.

Figure 4B is a graphic representation depicting the

25   effect of NGF on Bcl-2 expression in MM4 cells sham irradiated.

Figure 4C is a photograph of a Western blot depicting the effect of NGF on Bcl-2 expression in MM4 cells UV-irradiated or sham irradiated.

30   Figures 5A and 5B are photomicrographs showing the high levels of p75 NGF-R expression in melanocytes and bulbar keratinocytes of the outer root sheath in the lower portion of anagen hairs.

Figure 5C is a photomicrograph showing p75 NGF-R levels in melanocytes and keratinocytes of telogen hairs.

Figures 5D and 5E are photomicrographs showing p75 NGF-R levels in melanocytes and keratinocytes in anagen

5 hairs of patients with alopecia areata.

Figure 6 is a photomicrograph showing the effect of nerve growth factor on BCL-2 induction in melanocytic cells transfected with p75 NGF-R.

Figure 7 is a bar graph depicting the downregulation

10 of p75 NGF-R abrogates NGF effect on melanocyte survival. Cells were transfected with p75 NGF-R oligonucleotides and NGF-R expression was documented by indirect immunofluorescence.

Figure 8 is a bar graph depicting that BCL-2

15 downregulation abrogates NGF protective effect on UV irradiated melanocytic cells.

Figure 9A is a photomicrograph showing the effect of UV irradiation and nerve growth factor supplementation on keratinocyte apoptosis.

20 Figure 9B is a bar graph depicting the effect of UV irradiation and nerve growth factor supplementation on keratinocyte survival.

Figure 10 is a photomicrograph showing the effect of nerve growth factor depletion of BCL-2 level in

25 keratinocytes.

Figure 11 is a graph of experimental results showing competition for binding to p75^{NTR} by CKGAIC (SEQ ID Nos.) peptide and β-amyloid.

Figure 12A is a bar graph depicting abrogation of β-

30 amyloid mediated apoptosis by a KGA-containing decapeptide.

Figure 12B is a bar graph depicting abrogation of β-amyloid mediated apoptosis by a KGA-containing decapeptide.

Figure 12C is a bar graph depicting abrogation of β-amyloid mediated apoptosis by a KGA-containing hexapeptide.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is based on Applicants' finding that basal layer melanocytes and keratinocytes undergo programmed cell death, or apoptosis. Specifically,

5   Applicants have demonstrated that melanocytes and keratinocytes of the basal layer of the epidermis and the hair follicle undergo apoptosis. Apoptosis is an active process of self-destruction that occurs in vertebrate cells. Apoptosis follows a distinct pattern of events

10   characterized by plasma membrane blebbing, cell volume contraction, nuclear pyknosis and inter nucleosomal DNA degradation following the activation of Ca^+/Mg^{2+} dependent endonucleases. (Hockenberry, D. M., et al., Cell 75:241-251 (1993); Garcia, I., et al., Science 258:302-304

15   (1992)). Apoptosis is a highly conserved mechanism among species. Cells carry in their nuclei a genetic program for apoptosis, that can be activated upon the proper triggering, such as in response to changes in levels of hormones or growth factors in the cellular environment.

20   (Allsopp, T. E., et al., Cell 295-307 (1993); Barinaga, M. et al., Science 259:762-763 (1992); Barinaga, M., et al., Science 263:754-755 (1994)). The "apoptotic" genes encode proteins which will induce apoptosis. However, recent evidence suggests that cells that do not undergo apoptosis

25   express protective proteins, one of which is Bcl-2, which interact with the apoptotic proteins, sequester them and prevent their activity (Allsopp, T. E., et al., Cell 295-307 (1993)). It thus appears that a mechanism exists to protect cells from apoptosis.

30   To examine if UV-induced melanocyte death is apoptotic, cultures of pure human epidermal melanocytes or the human melanoma cell line MM4 (provided by Dr. U. Stierner, Goteborg, Sweden) were exposed to 5, 10 or 25 mJ/cm^2 UV irradiation, doses well within the physiologic UV

range that reaches the basal layer of the epidermis during casual sun exposure. (See Example 1). Sham irradiated control cultures were handled identically but placed under a dark cloth adjacent to the UV beam. After 1-3 daily

5   irradiations, many cells were detaching from the dish surface (See Figures 1A and 1C), while the majority of the cells in sham irradiated control cultures appeared healthy (See Figures 1B and 1D).

Total cellular DNA isolated from paired UV-irradiated

10 cultures displayed the characteristic endonuclease-induced DNA fragmentation into multimers, the so-called DNA ladder, while DNA of sham irradiated controls was not fragmented (Figure 1E). Duplicate UV-irradiated cultures stained with propidium iodide displayed the characteristic compaction,

15 margination and fragmentation of nuclear chromatin, as well as homogenous nuclear staining (Figures 1F and 1G). In sham irradiated cultures, fewer than 6% of the cells stained positively with propidium iodide. In contrast, approximately 30% and 60% of cells irradiated with 10 and

20 25 mJ/cm^2 respectively were propidium iodide positive (Figure 1H). These data strongly suggest that UV irradiation induces apoptotic death in cells of melanocytic origin.

However, melanocytes in vivo are not known to undergo

25 apoptosis after UV-irradiation. As described herein, Applicants have demonstrated that these cells have a mechanism necessary to protect them from apoptotic cell death.

It had previously been shown that both the high

30 affinity and low affinity nerve growth factor receptors, 140 kD and trkA and p75 NGF-R, were expressed in vitro on the surface of appropriately stimulated human melanocytes. (Peacocke, M., et al., Proc. Natl. Acad. Sci. U.S.A. 85:5282-5286 (1988); Yaar, M., et al., Clin. Res. 40:531A

(1992)). It had also been shown that keratinocytes express nerve growth factor. (Yaar, M., et al., J. Cell Biol. 115:821-828 (1991); DiMarco, E., et al., J. Biol. Chem. 266:21718-21722 (1991)).

5   Applicants now describe herein, that nerve growth factor enhances the survival of human melanocytes after injury, for example, due to ultraviolet light exposure or growth factor deprivation.

       Cultured human melanocytes were exposed to a solar
10 simulator (5, 10, 25 mJ/cm^2 UVB dose) or sham irradiated as described in Example 1 and then maintained in suboptimal serum-free medium, and continuously provided with either 50 ng/ml nerve growth factor or diluent alone. (See Example 2). After UV irradiation, the majority of melanocytes and
15 MM4 cells not supplemented with NGF were detaching from the dish surface. (See Figures 2F and 2G). In contrast, cultures supplemented with NGF appeared healthy. (See Figures 2H and 2I).

       Cell yields of melanocytes (Figure 2A) and MM4 cells
20 (Figures 2B and 2C) irradiated with 10 mJ/cm^2 and supplemented with 50 ng/ml NGF were significantly higher than those of cells supplemented with diluent alone (melanocytes: 7 experiments $p < 0.0085$; MM4 cells: 4 experiments $p < 0.0001$, ANOVA). Furthermore, supplementation
25 with basic fibroblast growth factor (bFGF), a major mitogen for cells of melanocytic origin (Halaban, R., et al., In Vitro Cell Devel. Biol. 23:47-52 (1987); Halaban, R., et al., J. Cell Biol. 107:1611-1619 (1988)), failed to improve MM4 cell survival after UV irradiation despite its
30 mitogenic effect on sham irradiated cells (Figures 2D and 2E).

       To explore the mechanism of the striking response of UV irradiated cells to NGF, paired cultures were irradiated with UVB light (5, 10 or 25 mJ/cm^2 UVB dose), or sham

-14-

irradiated, and then incubated with antibodies to the high affinity component of the NGF receptor, trk. Melanocytes in UV-treated cultures displayed more trk receptors than sham irradiated controls. Northern blot analysis checking

5 the mRNA levels of the p75 NGF-R showed several-fold higher transcript levels in NGF-supplemented melanocytes than in diluent controls.

To determine if melanocytes undergo p75 NGF-R mediated apoptotic cell death after UV irradiation, melanocytes were

10 exposed to UVB (10 or 25 mJ/cm^2) or were sham irradiated, as described in Example 1, then maintained in suboptimal serum-free medium. Both UVB irradiation and suboptimal culture conditions, previously shown to induce p75 NGF-R expression on melanocytes, induced the DNA fragmentation

15 patterns classic for apoptosis.

To determine whether NGF can rescue injured melanocytes from apoptosis, duplicate cultures were irradiated as described above, and maintained in medium containing 50 ng/ml NGF or diluent alone. Irradiated

20 cultures not supplemented with NGF showed the characteristic DNA fragmentation, while cultures supplemented with NGF showed far less fragmentation (Figure 3A). As described in Example 3, within twenty-four hours, in NGF-treated versus control melanocytes, 12 % versus 30 %

25 of nuclei showed fragmentation (p less than 0.05, paired test). (Figure 3B). Cell yields and thymidine labeling index determined daily for 19 days were higher in NGF-treated cultures (p less than 0.001), up to 6.5-fold and 10-fold, respectively.

30 To determine if melanocyte apoptosis is mediated by p75 NGF-R, cultures were treated as above, and then incubated in the presence of a blocking anti-human p75 NGF-R monoclonal antibody believed to act as a pseudo-ligand for the p75 NGF-R. (Anti-human p75 NGF-R monoclonal

antibody courtesy of Moses V. Chao, Cornell University Medical Center, New York, NY; Ross, et al., Proc. Natl. Acad. Sci. 81:6681 (1984)). Like NGF, the antibody suppressed melanocyte apoptosis in UV-irradiated cultures,
5 while anti-rat p75 NGF-R antibody that did not bind the human p75 NGF-R had no effect.

Northern blot analysis of melanocyte RNA from donors of different ages showed that p75 NGF-R was higher in older donors, while in contrast the level for other growth factor
10 receptors was unchanged or decreased with age, suggesting a greater vulnerability to apoptosis with aging, consistent with the clinical tendency for older persons to experience progressive hair loss.

Thus, one embodiment of the present invention relates
15 to a method of preventing or inhibiting melanocyte cell loss after injury. The melanocytes are located in the basal epidermal layer and include melanocytes located in the skin and in hair follicles. The type of injury includes injury due to exposure to ultraviolet light,
20 especially UVB, for example, in habitually sun-exposed skin, and injury due to the normal aging process. Injuries can also include disease conditions such as alopecia areata.

More specifically, the invention relates to methods of
25 preventing, or inhibiting, apoptosis in melanocytes and keratinocytes. As described above, Applicants have shown that apoptosis in melanocytes is mediated by the p75 NGF receptor. If the receptor is occupied, that is, if the receptor has bound an appropriate ligand, apoptosis is
30 inhibited in the cell.

An example of an inappropriate ligand is aggregated The Alzheimer's disease associated protein, β-amyloid. Binding of β-amyloid to the p75^{NTR} activates the receptor and results in apoptotic cell death of the melanocytes.

Applicants demonstrate herein that supplementation of normal human melanocytes with β-amyloid 1-40 peptide at low concentrations ($\leq 1 \mu M$) leads to extensive outgrowth of dendrites, the melanocyte cell processes analogous to

5 neurites of neurons, without decreasing cell yields. At higher concentrations of β-amyloid peptide, melanocyte cell yields decrease progressively and the remaining cells appear unhealthy. Additionally, in these cultures there is focal development of plaque-like structures consisting of

10 aggregated dying melanocytes, similar to the in vivo "senile plaques" observed in patients with AD.

Applicants further demonstrate herein that maintaining melanocytic cell cultures in ≥ 25 μM β-amyloid 1-40, compared to control cultures, significantly increases the

15 proportion of apoptotic cells and upregulates Bax protein expression approximately three fold. Recent *in vivo* and *in vitro* data suggest that the β-amyloid induced-neuronal death exhibits classical characteristics of programmed cell death or apoptosis (Cotman, C.W. and Anderson, A.J., Mol.

20 Neurobiol., 10:19-45 (1995); Su, J.H., et al., Neuroreport, 5:2529-2533 (1994). The molecular pathways that regulate apoptosis in neurons have been identified in part. Evidence suggests that the product of the proto-oncogene Bcl-2 delays the onset of apoptosis in neurons that are

25 dependent for survival on neurotrophic factors (Allsopp, T.E., et al., Cell, 73:295-307 (1993); Garcia, I., et al., Science, 258:302-304 (1992). Conversely, overexpression of a 21 kD Bcl-2-associated protein, Bax, accelerates apoptotic death of cells (Oltvai, Z.N, et al., Cell,

30 74:609-619 (1993)).

Melanocytes express both the low affinity p75[NTR] and high affinity 140 kD trk A (p140[trk A]) receptors for NGF and providing NGF to melanocytes results in activation of the p140[trkA] pathway, presumably through co-ordinate binding of

$p140^{trk\ A}$ and multiple $p75^{NTR}$ molecules as postulated to occur in neurons exposed to NGF, that in turn activates an intracellular signal transduction pathway leading to enhanced expression of Bcl-2 and enhanced cell survival.

5   Applicants further demonstrate herein that β-amyloid binds competitively to the $p75^{NTR}$. Studies previously published suggest that the specific binding site for the $p75^{NTR}$ is amino acids 29-36 of the resulting NGF protein (Ulrich, A., et al., Nature 303:821-825 (1983), and that if

10   the sequence lysine-glycine-lysine (residues 32-34 of NGF) is changed to lysine-glycine-alanine, the peptide has approximately half the affinity for the receptor as native NGF. Amino acid residues 28-30 of the β-amyloid protein are lysine-glycine-alanine. Furthermore, computerized

15   structure analysis of β-amyloid reveals that these amino acids have a high probability of being in a loop turn of the protein, suggesting a high probability that this β-amyloid peptide sequence plays a role in receptor binding.

A cyclic decapeptide was therefore synthesized by

20   attaching two cysteine residues to the beginning and the end of the β-amyloid fragment consisting of amino acids 24-31: VGSNKGAI (SEQ ID NO: 1). Cold peptide competitively inhibited ^{125}I-β-amyloid binding, with 50% inhibition occurring at 25 nM. Furthermore, β-amyloid 200 nM reduced

25   by cell yields by ~60% (p<.02), but this cell loss was blocked by the peptide (200 nM). Peptide alone had no effect on cell yield. These findings indicate that apoptosis of neurons in Alzheimer's Disease results from the interaction of β-amyloid with $p75^{NTR}$. The data also

30   suggest that β-amyloid-mediated death of neurons may be prevented by delivery of a synthetic peptide that blocks the β-amyloid binding sites.

Binding of NGF to $p75^{NTR}$ is mediated through amino acid residues 29-36, TDIKGKEV (SEQ ID NO: 2), that are part of

the β-hairpin loop of NGF (Ibánez, C.F., *et al.*, <u>Cell</u>, 69:329-341 (1992)). If lysine (K) at position 34 is replaced by alanine (A), the resulting mutant NGF molecule still binds p75^{NTR} but with 50% lower affinity.

5   Interestingly, in β-amyloid the amino acid residues 28-30, which are present in both the 1-40 and the 25-35 β-amyloid peptides, are KGA, a sequence that appears to permit p75^{NTR} binding by β-amyloid. Computerized structure analysis of β-amyloid suggests that the KGA residues have a high

10 probability (>60%) of being in a loop turn, the highest probability of any portion of this 40 amino acid peptide, suggesting that this sequence constitutes a binding site for p75^{NTR}. Furthermore it was reported that expression of p75^{NTR} enhances the toxic effect of β-amyloid on cells,

15 possibly through binding and activation of the receptor.

Based on the above data, it is reasonable to believe that the specific three amino acid sequence lysine-glycine-alanine (KGA) in the β-amyloid protein binds the 75 kD transmembrane neurotrophin receptor on CNS neurons,

20 activating the programmed cell death pathway, mediated in part by an increase in intracellular Bax levels.

It is also reasonable to believe that competitively inhibiting the binding of β-amyloid peptide blocks this aberrant receptor activation and the resulting apoptosis.

25 For example, providing full-length NGF, or a biologically active fragment, analog, derivative, variant or mutant thereof results instead in preferential binding of p75^{NTR} coordinately with p140^{trkA} binding, leading to activation of a second signal transduction pathway that results in

30 neuronal cell survival.

The term "biological activity" of NGF, or a fragment, derivative, analog, variant or mutant NGF, is defined herein as the activity of the NGF to specifically bind to the p75^{NGF} receptor. Such activity can be measured by the

methods described herein, or by other methods known to those skilled in the art. Another biological activity of an NGF fragment, analog, derivative, variant or mutant is the antigenic property of inducing a specific immunological

5 response as determined using well-known laboratory techniques. For example, a biologically active NGF fragment can induce an immunological response which produces antibodies specific for the NGF (anti-NGF antibodies).

10 Mammalian NGF is a protein, consisting of three subunits, α, β, and γ, which interact to form an approximately 130 kD complex. (Ulrich, A., *et al.*, *Nature* 303:821-825 (1983)). However, all known effects of NGF are mediated by the 26 kD β-subunit through its receptor.

15 There are two types of NGF receptors, one of a low molecular weight of approximately 75 kD, and the other of a higher molecular weight of approximately 140 kD. Both are believed necessary for the high affinity binding of NGF which is necessary for cellular response. The higher

20 molecular weight receptor was recently found to be the protooncogene, trk, which is a member of the tyrosine kinase family. (Yaar, M., *et al.*, *J. Cell Biol.*, 115:821-828 (1991); Chao, M., *et al.*, *Science* 232:518-521 (1986); Klein, R.S., *et al.*, *Cell* 65:189-197 (1991)). NGF has been

25 sequenced and cloned as described in Ulrich, A., *et al.*, *Nature*, 303:821-825 (1983), the teachings of which are herein incorporated by reference. Thus, the entire NGF protein complex, one of its active subunits, such as the 26 kD subunit, or any biologically active fragment of NGF can

30 be used to occupy the receptor. The biological activity of an NGF protein fragment can be determined by *in vitro* bioassay, for example, as described in DiMarco, E., *et al.*, *J. Biol. Chem.*, 266:21718-21722 (1991), the teachings of which are herein incorporated by reference.

Other substances that mimic NGF can act as a pseudo-ligand for the receptor. For example, the anti-human p75 NGF-R antibody described in Ross, et al., Proc. Natl. Acad. Sci. 81:6681 (1984) binds to p75 NGF-R and suppresses

5 apoptosis in melanocytes. Other pseudo-ligands are KGA-containing peptides that bind p75^{NTF}. Examples of such pseudo-ligands are the cyclic KGA-containing hexapeptides and decapeptides described, for example, in SEQ ID NOs.4, 9 and 10. These substances include other neurotrophic

10 factors and neurotrophins, such as NT-3, -4, and -5, which are also capable of binding to the p75 NGF-R. (DiMarco, E., et al., J. Biol. Chem., 268:24290-24295 (1993); Yaar, M., et al., J. Invest. Derm., 100:554 (1993)). Additional substances, either protein or chemical in nature, can be

15 produced and evaluated for their NGF-R binding ability. For example, a chemical substance can be produced that mimics the composition of NGF. This substance can be evaluated as described above for NGF activity.

Alternatively, a method of preventing epidermal

20 melanocyte cell loss can encompass downregulating the expression of the p75 NGF-R on epidermal melanocytes. This would also result in fewer unoccupied receptor molecules and hence, suppress apoptosis and prevent melanocyte cell loss. Downregulation can be accomplished, for example, by

25 introducing into the melanocyte a substance that inhibits or decreases the transcription of the gene encoding the p75 NGF-R. For example, an antisense oligonucleotide which is complementary to the cellular mRNA encoding the p75 NGF-R can be introduced into the melanocyte in such a manner that

30 the antisense oligonucleotide hybridizes with the mRNA, thereby preventing translation of the mRNA into p75 NGF-R protein.

Alternatively, epidermal melanocytes can be contacted with a substance which binds to p75 nerve growth factor

receptor expressed on the surface of the melanocytes. The substance, for example, can be nerve growth factor in a pharmaceutically acceptable carrier or an antibody capable of binding to p75 nerve growth factor and acting as a

5   pseudo-ligand. Pseudo-ligands include substances that mimic nerve growth factor, such as, e.g., peptides, the KGA containing peptides described herein, organic molecules, antibodies and antibody fragments.

Pseudo-ligand antibodies which can be used in the

10 present invention are capable of binding to p75 nerve growth factor receptor. The term antibody is intended to encompass both polyclonal and monoclonal antibodies. The preferred pseudo-ligand antibody is a monoclonal antibody reactive with a p75 nerve growth factor receptor. The term

15 antibody is also intended to encompass mixtures of more than one antibody reactive with a p75 nerve growth factor receptor (e.g., a cocktail of different types of monoclonal antibodies reactive with a p75 nerve growth factor receptor). The term antibody is further intended to

20 encompass whole antibodies, biologically functional fragments thereof, and chimeric antibodies comprising portions from more than one species, bifunctional antibodies, etc. Biologically functional antibody fragments which can be used are those fragments sufficient

25 for binding of the antibody fragment to the p75 nerve growth factor receptor to occur.

The chimeric antibodies can comprise portions derived from two different species (e.g., human constant region and murine variable or binding region). The portions derived

30 from two different species can be joined together chemically by conventional techniques or can be prepared as single contiguous proteins using genetic engineering techniques. The portions derived from two different species can also be produced by recombinant means and then

joined as described above. DNA encoding the proteins of both the light chain and heavy chain portions of the chimeric antibody can be expressed as contiguous proteins or can be produced by recombinant means and joined using

5 techniques known to those of skill in the art.

One mechanism by which p75 induces apoptosis may involve the cellular ratio of p75^{NTR} to TRK in combination with the level of NGF. The abundance of NGF, the neurotrophin that presumptively enhances the survival of

10 cells that express both of its receptors, decreases with age (Larkfors, L. *et al*. Brain Res. 1987; 427:55-60). In addition, the Applicants have found a prominent increase in the experssion of the apoptotic p75^{NTR} with aging (Yaar, M. *et al.*, J. Invest Deratol., 1997;108:568). These data

15 suggest that a relative lack of NGF and/or increased levels of an apoptosis-inducing ligand such as β-amyloid may lead to apoptotic loss of cells.

The end result of p75 NGF-R binding to its ligand is the expression of the protective protein, Bcl-2. Bcl-2 has

20 been shown to prevent some classes of cell death in lymphocytes and neurons. (Veis, D.J., *et al.*, Cell 75:229-240 (1993)). As described in Example 4, Applicants have now shown the expression of Bcl-2 by injured melanocytes after treatment with NGF. Apoptosis can be inhibited by

25 the expression of the protective protein, Bcl-2. Thus, another method of preventing melanocyte cell loss comprises a method of upregulating expression of the Bcl-2 protein in melanocytes. This can be accomplished, for example, by inserting a nucleotide sequence encoding Bcl-2 into an

30 expression vector capable of expressing the encoded Bcl-2 in vertebrate cells. Such an expression vector can be constructed, for example, as described in Allsopp, T.E., *et al.*, Cell 73:295-307 (1993), the teachings of which are herein incorporated by reference. This Bcl-2 expression

vector can then be introduced into melanocytes using standard laboratory techniques, such as, for example, microinjection, calcium-phosphate precipitation, or microprojectible bombardment.

5   Alopecia areata (AA) is a common disease of the hair follicle, affecting about 2% of new patients attending dermatology clinics in the United States and in Britain (Price, V.H., J. Invest. Dermatol., 96:685 (1991)). In alopecia areata, the hair follicle, in response to some

10 unknown signal or injury, is suddenly precipitated into premature telogen, and then cycles in a shortened aborted cycle in which it is repeatedly arrested part way through early anagen. The follicle may remain in this arrested state but is capable of resuming normal growth after months

15 or years. The nature of the signal or injury and the anatomical target for this abnormality are unknown.

Histologically, AA is characterized by peribulbar lymphocytic infiltrate of predominantly T helper cells (Lever, W.F. and Schaumburg-Lever, G., eds., HISTOPATHOLOGY

20 OF THE SKIN, J.B. Lippincott Co., Philadelphia, PA, 1990, pp. 223-224), strongly suggesting the involvement of the cellular immune system perhaps through a loss of discrimination of self and non-self antigens (Goldsmith, L.A., J. Invest. Dermatol., 96:985-1005 (1991)).

25 Alternatively, an intrinsic abnormality in the follicular keratinocyte could be activated under the influence of internal or external triggers which eventually may lead to cellular degeneration and peribulbar inflammatory infiltrate. However, to date no specific antigen has been

30 identified to support the autoimmune theory and no specific intrinsic difference has been reported between normal bulbar and AA keratinocytes.

As described in Example 5, indirect immunofluorescent studies were performed on biopsy material obtained from

normal subjects and alopecia areata patients in an effort
to detect differences in the NGF signaling system during
conditions characterized by keratinocyte and melanocyte
death. Results show high levels of p75 NGF-R in melanocytes
5   and bulbar keratinocytes of the outer root sheath in the
lower portion of anagen hairs (Figures 5A and 5B),
suggesting a role for p75 NGF-R in hair growth. p75 NGF-R
levels were significantly reduced or p75 NGF-R was absent
melanocytes and keratinocytes of telogen hairs (Figure 5C).
10   Furthermore, in melanocytes and keratinocytes in anagen
hairs of AA patients, significantly lower levels of p75
NGF-R was also detected (Figures 5D and 5E), indicating
that decreased levels of this receptor may be involved in
the pathogenesis of AA by precipitating anagen hairs into
15   early telogen.

These findings strongly suggest that loss of the p75
NGF-R may lead to bulbar keratinocyte apoptosis and shift
the hair follicle towards telogen. Decreased p75 NGF-R in
anagen hair of AA patients may be the initial insult which
20   triggers telogen effluvium in these patients.

Thus, in another embodiment of the present invention,
the invention relates to a method of inducing hair growth
in a vertebrate. This is especially useful to delay or
prevent hair loss in humans, for example, in male pattern
25   baldness. Hair growth is induced by maintaining hairs in
the anagen phase, and preventing the initiation of the
telogen phase. As described above, p75 NGF-R levels were
significantly reduced, or absent in alopecia areata
patients. Thus, it is reasonable to believe that if the
30   level of NGF-R expression on the surface of hair follicle
keratinocytes is increased, the hairs are maintained in the
anagen phase resulting in hair growth. Upregulating the
expression of the p75 NGF-R can be accomplished by
inserting a nucleotide sequence encoding the p75 NGF-R into

an expression vector capable of expressing the encoded receptor protein in a vertebrate cell and introducing the receptor vector into the keratinocyte, resulting in expression of the encoded receptor. p75 NGF-R expression

5   vectors can be constructed as described in, e.g., Rabizadeh, S., et al., Science 261:345-348 (1993); Morgenstern, J.P., et al., Nucleic Acids Res. 18:3587 (1990). This p75 NGF-R expression vector can be introduced into keratinocytes using standard laboratory techniques,

10   such as, for example, microinjection, calcium-phosphate precipitation, or microprojectile bombardment. The cDNA sequences for human, rat and chicken p75 NGF-R are known. (Johnson, D., et al., Cell 47:545-554 (1986); Radeke, M. et al., Nature 325:593-597 (1987) and Large, T.H., et al.,

15   Neuron 2:1123-1134 (1989); Huer, J.G., et al., Devl. Biol. 137:287-304 (1990), respectively, the teachings of which are incorporated by reference).

Alternatively, a substance can be introduced into epidermal keratinocytes that upregulates the expression of

20   the p75 NGF-R, such as a transcription factor that promotes the transcription of the gene encoding the p75 NGF-R.

As discussed above, unoccupied p75 NGF-R results in apoptosis in melanocytes. Based on the data presented herein, Applicants reasonably expect that p75 NGF-R/NGF

25   mediated apoptosis also occurs in epidermal keratinocytes. Thus, the binding of p75 NGF-R to ligand in epidermal keratinocytes results in the expression of the anti-apoptotic protein, Bcl-2. Another method of inducing hair growth encompassed by the present invention relates to

30   upregulating the expression of Bcl-2 in epidermal keratinocytes. Upregulation of Bcl-2 expression can be accomplished by expressing the encoded Bcl-2 protein in keratinocytes in a similar manner as the expression of Bcl-2 protein in melanocytes as discussed above.

As discussed above, in biopsies from patients with AA, p75 NGF-R expression in keratinocytes of anagen hairs is significantly reduced or totally absent. In AA, the p75 NGF-R can be bound in vivo by a pathogenic autoantibody

5 that precludes further binding of commercial antibodies. To pursue the possibility that reduced levels of p75 NGF-R in AA are the result of a bound autoantibody, direct immunofluorescent studies can be performed on cross section from AA patients to determine if human immunoglobulins are

10 bound in areas known to express p75 NGF-R.

Another embodiment of the present invention relates to methods of inducing hair coloration in a vertebrate comprising inhibiting apoptosis in epidermal melanocytes. Epidermal melanocytes produce melanin pigment in organelles

15 called melanosomes and transfer the pigment to surrounding keratinocytes via extensive dendrites. Melanin pigmentation is the principal determinant of hair and skin color. Inhibiting apoptosis in melanocytes results in persistently pigmented keratinocytes, or hair coloration,

20 and thus, delays or prevents hair greying which is due to loss of hair bulb melanocytes.

Methods of therapy include administering to the individual a substance, e.g., the tripeptide KGA, or an analog thereof, in a manner which permits contact of the

25 substance with neurons of the CNS. For example, the pentapeptide CKGAC (SEQ ID NO: 3), or an analog thereof, can be chemically synthesized by methods well-known to one of skill in the art. The cysteine residues flanking the ends of the pentapeptide can be linked, e.g., by a

30 disulfide bond, to maintain the conformation required for binding of the peptide to the p75^{NTR}, thus inhibiting, or preventing apoptosis. The length of the peptide can be longer than a pentapeptide, as long as the KGA, or analog peptide is maintained in a configuration suitable for

binding activity. For example, as described herein, cyclic peptides have been made with the amino acid sequences and CVGSNKGAIC (SEQ ID NO: 4) these peptide compete for $p75^{NTR}$ binding with β-amyloid peptide.

5   Conversely, as a result of Applicants' discovery of the mechanism of apoptotic cell death in melanocytes and keratinocytes, methods are also provided that promote, apoptosis in these cells resulting in cell death. The promotion of cell death in keratinocytes may be desirable

10 to decrease, or completely inhibit hair growth in specific areas on an individual. For example, the inhibition of facial hair growth, forearm hair growth or leg hair growth is often desirable.

     Such inhibition of hair growth can be accomplished,

15 for example, by the use of a blocking antibody that will block the binding of NGF to the p75 NGF-R expressed on keratinocytes. The blocking antibody (or an antibody fragment or peptide) will bind to the p75 NGF-R and thus prevent NGF from binding to the NGF-R. Thus, the NGF/p75

20 NGF-R mediated anti-apoptotic pathway is inhibited and cell death will be permitted, or enhanced after injury to the cells. For example, the specific area in which hair growth is to be inhibited can first be irradiated with UV light and then a composition comprising the blocking antibody can

25 be applied (e.g., in a cream or ointment), resulting in apoptosis of injured keratinocytes and inhibition of hair growth.

     In another embodiment of the present invention, the invention relates to in vitro methods of identifying novel

30 substances, capable of inducing hair growth or hair coloration or inhibiting hair growth in an individual. These methods can be based on Applicants' discovery of the apoptotic mechanism of death in melanocytes and

keratinocytes. An *in vitro* method of evaluating p75 NGF-R/NGF mediated apoptosis can use, for example, C57BL-6 mouse skin specimens with synchronized hair follicles either in telogen or anagen, as described in Paus, R., *et al.*, *Br. J. Dermatol.* 122:777-784 (1990), the teachings of which are incorporated herein by reference. These skin specimens, being larger than biopsies obtained from people, and having follicles in defined portions of the growth cycle are useful to investigate the relationship between NGF/NGF-R and growth state of the hair follicle. The necessary murine probes (cDNA and antibodies) are available. For example, anti-rat p75 NGF-R antibody is available from Accurate Chemical & Scientific Company (New York) and anti-mouse NGF antibody is available from Boehringer Mannheim Biochemicals (Indianapolis, IN). Rat NGF cDNA is described in Maisonpierre, P.C., *et al.*, *Science* 247:1446-1451 (1990) and rat p75 NGF-R cDNA is described in Radeke, M.J. *et al.*, *Nature* 325:593-597 (1987). A substance to be tested for anti-apoptotic activity in melanocytes can be evaluated in this, or another a melanocyte cell culture assay (e.g., as described in Example 1). Skin specimens or melanocytes can be maintained under conditions suitable for their proliferation and then exposed to UV irradiation. After irradiation, the substance to be tested for apoptotic activity can be added to the culture system. Subsequently, the cultured cells can be evaluated to determine whether cell death has been inhibited, or decreased.

Substances identified in this method are substances that specifically alter the apoptotic mechanism in melanocytes and kerantincytes. For example, substances that mimic nerve growth factor can be tested in an assay such as described above to evaluate their activity in inhibiting apoptosis. Additionally, substances identified

and evaluated by this method can be peptides, organic molecules, small organic molecules, antibodies or antibody fragments.

Substances identified using methods described herein,
5 found to bind p75 nerve growth factor receptor, or otherwise affect p75 nerve growth factor receptor, or found to initiate Bcl-2 expression, can be used in methods to induce hair growth, hair color or skin color. These methods comprise contacting epidermal cells, including
10 basal layer melanocytes or follicular keratinocytes, of a vertebrate with an effective amount of a substance capable of inducing hair growth, hair color or skin color by inhibiting apoptosis in melanocytes or keratinocytes. An effective amount of such an identified substance is an
15 amount effective to significantly decrease or completely inhibit apoptotic cell death in melanocytes and keratinocytes. The decrease of inhibition of apoptosis in melanocytes and keratinocytes can be evaluated using the methods described herein.

20 Various delivery systems are known and can be used to administer effective amounts of substances, such as naturally-occurring ligand or pseudo-ligand for p75 nerve growth factor receptor to inhibit apoptosis in melanocytes and keratinocytes. For example, encapsulation in
25 liposomes, microparticles, microcapsules, expression by recombinant cells, receptor-mediated endocytosis, construction of a naturally-occurring or pseudo-ligand encoding nucleic acid as part of a retroviral or other vector can be used. In one embodiment, a liposome
30 preparation can be used. The liposome preparation can be comprised of any liposomes which penetrate the stratum corneum and fuse with the cell membrane, resulting in delivery of the contents of the liposome into the cell. For example, liposomes such as those described in U.S.

Patent No. 5,077,211 of Yarosh, U.S. Patent No. 4,621,023 of Redziniak _et al._ can be used.

Administration of the substances can also be, for example, by topical application to the epidermis of a

5 vertebrate, such as a human, in a quantity sufficient to suppress apoptosis and prevent melanocyte or keratinocyte cell loss. The substance can be admixed in a pharmacological topical carrier such as a gel, an ointment, a lotion, a cream, or a shampoo and will include such

10 carriers as water, glycerol, alcohol, propylene glycol, fatty alcohols, triglycerides, fatty acid esters, or mineral oils. Other possible topical carriers include, e.g., liquid petrolatum, isopropyl palmitate, polyethylene glycol, ethanol (95%), polyoxyethylene monolauriate (5%) in

15 water, sodium lauryl sulfate (5%) in water. Other materials such as anti-oxidants, humectants, viscosity stabilizers, and similar agents may be added as necessary.

In addition, in certain instances, it is expected that the substances can be disposed within devices placed upon,

20 in, or under the skin. Such devices include transdermal patches, implants, and injections which release the substance in such a manner as to contact the skin or hair follicle either by passive or active release mechanisms.

The delivery vehicle can also contain perfumes,

25 colorants, stabilizers, sunscreens, or other ingredients. The substance can be applied, for example, topically to the epidermis at regular intervals, such as once or twice daily, in a suitable vehicle and at an effective concentration. Application can also be in a vehicle which

30 specifically targets the appropriate cells (i.e., either epidermal melanocytes or epidermal keratinocytes). For example, a membrane marker specific for melanocytes, such as melanocyte stimulating hormone (MSH), can be incorporated into a liposome containing a substance that

inhibits or decreases the transcription of the gene
encoding the p75 NGF-R.

An effective amount of a substance that inhibits,
decreases, or promotes apoptosis can be administered to an
5   individual using any of the above-described methods. The
actual preferred amounts of a ligand to be administered
will vary according to the specific ligand being utilized,
the particular compositions formulated, the mode of
application, and the particular situs and vertebrate being
10   treated. The concentration of the ligand effective to
suppress apoptosis and to prevent epidermal melanocyte cell
loss or epidermal keratinocyte cell loss, or to promote
apoptosis, in a vertebrate, such as a human, can be
determined using known, conventional pharmacological
15   protocols.

The following examples more specifically illustrate
the invention and are not intended to be limiting in any
way.

EXAMPLE 1:   EFFECT OF UV IRRADIATION ON MELANOCYTIC CELL
20   DEATH

Melanocytes or MM4 cells were plated in 60 mm diameter
tissue culture dishes. Melanocytes were maintained in
Medium 199 supplemented with 7% fetal bovine serum (FBS),
10 ng/ml epidermal growth factor (Collaborative Research),
25   10 μg/ml insulin (Sigma), 10^{-9} M triiodothyronine
(Collaborative Research), 10 μg/ml transferrin (Sigma), 1.4
x 10^{-6} M hydrocortisone (Calbiochem), 10^{-9} M cholera toxin
(Calbiochem), and 10 ng/ml basic fibroblast growth factor
(Collaborative Research) (basal melanocyte medium). MM4
30   cells were maintained in 55.3% DME, 27.6% L15, 15% FBS, 1%
nonessential amino acids (GIBCO BRL), 2mM glutamine and 10
μg/ml insulin. After 24 hours, medium was replaced by
phosphate buffered saline (PBS) and cells were irradiated

-32-

using a 1 KW xenon arc solar simulator (XMN 1000-21, Optical Radiation Corp., Azuza, CA) with 5, 10, or 25 mJ/cm^2 UV through the petri dish plastic cover. Irradiance was adjusted to 4×10^{-5} UV cm^{-2} and metered with a research

5 radiometer (model IL1700A, International Light, Newburyport, MA) fitted with a UVB probe at 285 ± 5 nm. After UV irradiation, cells were maintained in their respective media without FBS for 2 days (MM4 cells) or 3 days (melanocytes) and processed as indicated. Sham

10 irradiated control cultures were handled identically but placed under a dark cloth adjacent to the UV beam.

Cells in 100 mm tissue culture dishes were washed with cold PBS and disrupted in lysis buffer pH8 (10 mM tris, 150 mM NaCl, 0.1 mM EDTA, 1% SDS, 200 μg/ml proteinase K).

15 After 15 hour incubation at 37°C, samples were extracted twice with phenol plus chloroform (1:1, V/V) and precipitated overnight with ethanol (2.5 x volume) and 3 M sodium acetate (1/10 x volume). The DNA was then digested with DNAse free ribonuclease (10 μg/ml) for one hour at

20 37°C, separated on 1% agarose gel and stained with ethidium bromide. The size marker is 100 bp DNA ladder (STD) (Gibco/BRL, Gaithersburg, MD). Figure 1E shows that DNA fragmentation occurs in UV-irradiated but not sham irradiated MM4 cells.

25 Melanocytes were cultured on 8 chamber tissue culture slides (Nunc Inc., Naperville, IL) and were UV irradiated with 10 mJ/cm^2 as above. Four μM of propidium iodide (PI) was added to melanocyte cultures 24 hours after irradiation, for 5 minutes at 37°C. Cultures were washed

30 with PBS and nuclei were analyzed using a Leitz confocal laser microscope (Leica, Deerfield, IL). Figure 1F shows fragmentation of nuclear chromatin of UV-irradiated melanocytes. Figure 1G shows homogenization of nuclear chromatin of UV-irradiated melanocytes.

Melanocytes were sham or UV irradiated with 10 mJ/cm^2 and 25 mJ/cm^2. Twenty-four hours after irradiation, 4 μM of propidium iodide was added to cultures as above and cells were viewed with fluorescent phase contrast Nikon

5 microscope. The number of fragmented or homogeneously stained nuclei versus nonstained nuclei was determined in several representative fields and expressed as a percent of total cells. A minimum of 130 cells were counted for each condition. Figure 1H shows the percent PI-positive cells

10 in melanocyte culture.

EXAMPLE 2: NERVE GROWTH FACTOR ENHANCES SURVIVAL OF HUMAN MELANOCYTES AFTER INJURY

Melanocytes were UV-irradiated three times on three consecutive days with 0, 5, 10 or 25 mJ/cm^2 doses. After

15 each UV exposure, cells were placed until the next irradiation in fresh melanocyte medium containing 50 ng/ml NGF or diluent alone. Figure 2A shows melanocyte yield after three daily UV irradiations of 0, 5, 10 and 25 mJ/cm^2.

20 Figures 2B and 2D show MM4 cell yields 24-72 hours after a single UV irradiation of 10 mJ/cm^2. Figures 2C and 2e show MM4 cell yields 24-72 hours after sham irradiation. Cells in Figures 2B and 2C were supplemented with 50 ng/ml NGF or diluent alone. Cells in Figures 2D and 2E were

25 supplemented with 50 ng/ml bFGF or diluent alone.

Figure 2F shows the cell morphology of melanocytes after UV irradiation daily for three days with 10 mJ/cm^2 and supplemented with diluent alone. Figure 2G shows the cell morphology of MM4 cells after UV irradiation once with

30 10 mJ/cm^2 and supplemented with diluent alone. Figure 2H shows the cell morphology of melanocytes after UV irradiation daily for three days with 10 mJ/cm and supplemented with 50 ng/ml NGF. Figure 2I shows the cell

morphology of MM4 cells after UV irradiation once with 10 mJ/cm^2 and supplemented with 50 ng/ml NGF.

EXAMPLE 3: NERVE GROWTH FACTOR RESCUES INJURED MELANOCYTES UNDERGOING APOPTOSIS

5   Melanocytes or MM4 cells were plated as described in Example 1. After irradiation, melanocytes were maintained in basal melanocyte medium lacking FBS and hydrocortisone, with 50 ng/ml NGF or diluent alone (melanocyte medium). MM4 cells were maintained in DME supplemented with 50 ng/ml

10 NGF or diluent alone.

Twenty-four hours after UV irradiation, cells supplemented with diluent alone (-) showed fragmentation, while DNA of cells supplemented with NGF (+) was not fragmented. The standard (STD) is 100 bp DNA ladder

15 (Gibco/BRL).   (See Figure 3A).

Melanocytes were irradiated with 10 mJ/cm^2 or were sham irradiated as in Example 1 and then provided 50 ng/ml NGF or diluent alone. Twenty-four hours after irradiation, approximately 30% of diluent treated cells but only 12% of

20 NGF supplemented cultures show positive nuclei. That is, propidium iodide staining was positive in approximately 30% of nuclei in cultures not supplemented with NGF but in only 12% positive nuclei in NGF-supplemented cultures. (See Figure 3B).

25 EXAMPLE 4: MELANOCYTE EXPRESSION OF BCL-2 PROTEIN AFTER UV INJURY

To determine if NGF induces Bcl-2 protein, MM4 cells were UV-irradiated with 10 mJ/cm^2 or sham irradiated and then supplemented with NGF or diluent alone as explained in

30 Example 3. Twenty-four hours after irradiation, cells were washed with PBS and then detached with 0.5 mM EDTA and washed again with PBS. 10^6 cells were incubated with 3.25

µg/ml mouse anti human Bcl-2 monoclonal antibody (DAKO Co., Carpinteria, CA) or with the same concentration of normal mouse IgG (Cappel, Organon Teknika Co., West Chester, PA, USA) in PBS with 0.3% Saponin (Sigma, St, Louis, MO) for 2

5 hours at 4°C. After three washes with PBS, cells were incubated with fluorescein-conjugated goat anti-mouse IgG (1 hour at 4°C) (Cappel), washed four times in PBS, fixed with 0.1% fresh formaldehyde, and washed three times in PBS. Fluorescence intensity was determined using FACScan

10 flow cytometer (Becton-Dickinson, San Jose, CA).

The results show that UV-irradiated (Figure 4A) or sham irradiated (Figure 4B) cells, cells supplemented with diluent alone, or sham irradiated cells supplemented with NGF had only low levels of Bcl-2 protein FACScan analysis.

15 However, the Bcl-2 level was substantially higher in cells subjected to UV irradiation followed by NGF supplementation (Figure 4A). (-) mouse IgG control; (⋯) diluent alone; (...) 50 ng/ml NGF.

Proteins from duplicate cultures analyzed by Western

20 blotting confirmed Bcl-2 induction in UV irradiated NGF supplemented melanocytic cells (Figure 4C). MM4 cells were extracted in RIPA buffer (50 mM Tris-HCl [pH 8.0], 0.15 M NaCl, 0.5% sodium deoxycholate, 1% Triton X-100) in the presence of one µg/ml aprotinin and 75 µg/ml

25 phenylmethylsulfonyl fluoride, sonicated for 1-3 seconds and centrifuged. 45 µg of protein per lane were separated on 12% SDS/PAGE and blotted onto nitrocellulose paper (overnight, 40V). Blots incubated with 3.25 µg/ml anti-human Bcl-2 antibody (DAKO) reveal a band at the reported

30 25 kDa molecular weight: (+) 50 ng/ml NGF, (-) diluent alone.

EXAMPLE 5: IMMUNOFLUORESCENT STUDIES

Punch biopsies (6 mm diameter) can be obtained from scalps of patients for example, with patchy AA, alopecia totalis, alopecia universalis as well as uninvolved sites of AA patients and age matched controls and snap frozen for

5 immunofluorescent studies. Immunostaining of fresh frozen tissues is compared with formaldehyde fixed tissues to determine if the antigen detection level is better in frozen sections. If the antibodies recognize formaldehyde fixed antigens with the same accuracy as non-denatured

10 antigen, formaldehyde fixed tissues can be used for the immunofluorescent studies.

Immunofluorescence was performed as described in Yaar, M., et al., Lab Invest. 58:157-162 (1988). Briefly, 4-μM thick vertical sections of biopsy samples were incubated

15 with the first antibody overnight at 4°C. The second antibody applied was the appropriate fluorescein isothiocyanate conjugated antibody: either goat anti-rabbit or anti-mouse IgG (Cooper Biomedical). The second antibody was incubated for 30 minutes. Quantitation was performed

20 by analysis of fluorescence intensity on the Leica Confocal microscope as described in Lu, K., et al., Proc. Natl. Acad. Sci. USA 89:3889-3893 (1992). Figures 5A and 5B show high levels of p75 NGF-R in melanocytes and bulbar keratinocytes of the outer root sheath in the lower portion

25 of anagen hairs. Figure 5C shows that p75 NGF-R levels were significantly reduced or absent melanocytes and keratinocytes of telogen hairs. Figures 5D and 5e show that p75 NGF-R levels were significantly lower or absent in melanocytes and keratinocytes in anagen hairs of AA

30 patients.

EXAMPLE 6: UPREGULATION OF P75 NGF-R IN NGF SUPPLEMENTED MELANOCYTIC CELLS UPREGULATES BCL-2 PROTEIN.

To determine the role of p75 NGF-R in mediating NGF survival effect in melanocytic cells, MM4 cells were transfected with 5 ug DNA of PCMV5A expression vector carrying the p75 NGF-R cDNA, as well as, with 1 ug plasmid

5   SV40 Neo carrying a neomycin resistant gene. Control cultures were transfected with 10 ug of SV40 Neo plasmid. Cultures were maintained in DME supplemented with 50-100 ng/ml G418 (geneticin), without serum, in the presence of 50 ng/ml NGF. Total cellular proteins were extracted in

10 RIPA buffer (50 mM Tris-HCI, [pH 8.0], 0.15 M NaCI, 0.5% sodium deoxycholate, 1% Triton X-100) in the presence of 1 ug/ml aprotinin and 75 ug/ml phenylmethylsulfonyl fluoride, sonicated for 1-3 seconds and centrifuged. Blots were incubated with 3.25 ug/ml anti human BCL-2 antibody (DAKO).

15   The results shown in Figure 6 demonstrate that melanocytic cells constitutively express the two known forms of the BCL-2 protein, BCL-2 alpha and BCL-2 beta. However, the levels of BCL-2 alpha and BCL-2 beta are substantially higher in NGF supplemented cells transfected

20 with p75 NGF-R as compared to NGF supplemented control cells, indicating that in the presence of NGF, higher levels of p75 NFG-R in cells contribute to their survival.

EXAMPLE 7: DOWNREGULATION OF P75 NGF-R IN NGF SUPPLEMENTED MELANOCYTIC CELLS ABROGATES NGF EFFECT ON CELLS

25   To further determine the role of p75 NGF-R in mediating NGF survival effect in melanocytic cells, 19 mer antisense and nonsense (scrambled) p75 NGF-R oligonucleotides were synthesized and were sulfurized to the phosphorothioate form. The antisense sequence was

30 directed against the 5' end of the human p75 NGF-R coding region (Johnson, D., *et al.*, Cell, 47:545-554 (1986)). The following sequences were used: Antisense 5'→3'

GGCACCTGCCCCCATCGCC (SEQ ID NO: 5); Nonsense 5'-3'
CTCCCACTCGTCATTCGAC (SEQ ID NO: 6) (negative control).

Melanocytes were maintained in Medium 199 supplemented
with 5% fetal bovine serum (FBS), 10 ng/ml epidermal growth

5 factor (Collaborative Research), 10 μg/ml transferrin
(Sigma), 1.4 x 10^{-6} M hydrocortisone (Calbiochem), 10^{-9} M
cholera toxin (Calbiochem, 10 ng/ml basic fibroblast growth
factor (Collaborative Research) (basal melanocyte medium).

Near confluent cells were irradiated using a 1 KW

10 xenon arc solar simulator (XMN 1000-21, Optical Radiation
Corp., Azuza, CA) with 20 mJ/cm^2 UV through the petri dish
plastic cover. Irradiance was adjusted to 4 X 10^{-5} UV cm^{-2}
and metered with a research 20 radiometer (model IL1700A,
International Light, Newburyport, MA) fitted with a UVB

15 probe at 285 $\pm$ 5 nm. After UV irradiation, cells were
trypinized and incubated in suspension at 37°C for 30
minutes with 10 uM antisense or nonsense p75 NGF-R
oligonucleotides.

After the initial 30 minute incubation cells were

20 plated in 35 mm dishes in basal melanocyte medium without
serum. Cells were supplemented with fresh oligonucleotides
every 12 hours for 48 hours. Cells were visualized by
phase contrast microscopy and pictures of representative
fields were obtained. Cell yields were determined by

25 counting cells in several representative fields. The
results shown in Figure 7 demonstrate that in the absence
of p75 NGF-R (antisense NGF-R), NGF does not have an effect
on melanocyte survival as compared to cells expressing p75
NGFR (sense NGF-R). This experiment confirms the role of

30 p75 NGF-R in mediating the new growth factor effects in
melanocytic cells.

EXAMPLE 8: BCL-2 DOWNREGULATION ABROGATES NGF PROTECTIVE
EFFECT ON UV IRRADIATED MELANOCYTIC CELLS

Purified phosphorothioate oligonucleotides were purchased from Quality Controlled Biochemicals, Inc. (Hopkinton, MA). 19 mer oligonucleotides were designed based on the published human BCL-2 sequence (Tsujimoto, Y.

5 and Croce, C.M., _Proc. Natl. Acad. Sci. USA_, 83:5214-5218 (1986)). The sequence chosen was directed against the 5' end of the coding region starting 4 bases before the methionine initiation site. Nonsense oligonucleotides were used as control. Sequences used (all written 5'→3'):

10 Antisense CCCAGCGTGCGCCATCCTT (SEQ ID NO: 7); Nonsense CTCCCACTCGTCATTCGAC (SEQ ID NO: 8).

MM4 cells were maintained in 60 mm diameter tissue culture dishes in 55.3% DME, 27.6% L15, 15% FBS, 1% nonessential amino acids (GIBCO BRL), 2 mM glutamine and 10

15 μg/ml insulin. Near confluent cells were UV irradiated with 10 mJ/cm^2. Immediately after irradiation cells were incubated with 10 uM antisense or nonsense BCL-2 oligonucleotides in suspension at 37°C for 30 minutes. Then cells were plated in tissue culture dishes in the

20 presence or absence of NGF (50 ng/ml). Cells were supplemented with fresh oligonucleotides every 12 hours. Cell yield and BCL-2 level were determined 48 hours after irradiation. Cell yield was determined by counting cells in at least three representative field per each condition.

25 Figure 8 shows the results of a Western blot demonstrating that in the presence antisense BCL-2 oligonucleotides BCL-2 levels are almost undetectable. Cell yields of UV irradiated cultures supplemented with NGF and nonsense oligonucleotides (white bar) are significantly higher as

30 compared to nonsense supplemented cultures provided with diluent alone (dotted bar) (p<0.007, ANOVA). Cell yields of NGF supplemented cultures treated with BCL-2 antisense oligonucleotides (black bar) are significantly lower than NGF supplemented cultures provided with nonsense

oligonucleotides (white bar) demonstrating complete abrogation of NGF effect on the cells (p<0.003, ANOVA). In diluent supplemented culture yields of nonsense treated cells (dotted bar) were significantly higher than antisense
5   treated cells (dashed bar) (p<0.004, ANOVA). Morphologic appearance of MM4 cells confirmed the numerical cell yield data. This experiment demonstrated that BCL-2 protein is required for melanocytic survival after UV irradiation and that NGF affects melanocytic cell survival by upregulating
10 their BCL-2 level.

EXAMPLE 9: EFFECT OF UV IRRADIATION ON KERATINOCYTE CELL DEATH

Keratinocytes were plated in 60 mm diameter tissue culture dishes in MCDB153 supplemented with epidermal
15 growth factor (0.1 ng/ml human recombinant), insulin (5 μg/ml), hydrocortisone (0.5 μg/ml, calcium (0.15 mM), bovine pituitary extract (BPE), (2 ml per 500 ml medium), gentamicin (50 μg/ml), and amphotericin-B (50 ng/ml) (karetinocyte basal medium). Preconfluent cells were
20 irradiated in phosphate buffered saline (PBS) using a 1 KW xenon arc solar simulator (XMN 1000-21, Optical Radiation Corp., Azuza, CA) with 15 or 25 mJ/cm^2 UV through the petri dish plastic cover as described in Example 7. After UV irradiation, cells were maintained in their basal medium 3
25 days and processed as indicated. Sham irradiated control cultures were handled identically but placed under a dark cloth adjacent to the UV beam.

Cells were washed with cold PBS and disrupted in lysis buffer pH8 (10 mM tris, 150 mM NaCl, 0.1 mM EDTA, 1% SDS,
30 200 μg/ml proteinase K). After 15 hour incubation at 37°C, samples were extracted twice with phenol plus chloroform (1:1, V/V) and precipitated overnight with ethanol (2.5 X volume) and 3 M sodium acetate (1/10 x volume). The DNA

was then digested with DNAse free ribonuclease (10 μg/ml) for one hour at 37°C, separated on 1% agarose gel and stained with ethidium bromide. The data show that DNA fragmentation, characteristic of apoptotic cell death,

5 occurs in UV-irradiated but not sham irradiated keratinocytes.

EXAMPLE 10: NERVE GROWTH FACTOR ENHANCES SURVIVAL OF HUMAN KERATINOCYTES AFTER INJURY

Keratinocytes were UV-irradiated as in Example 9.

10 After irradiation cells were placed in fresh keratinocyte medium containing 50 ng/ml NGF or diluent alone. DNA fragmentation was determined as in Example 9. Figure 9A shows that UV irradiated keratinocytes supplemented with diluent alone (-) display the characteristic DNA

15 fragmentation, while DNA of UV irradiated cells supplemented with NGF (+) is not fragmented. The standard (STD) is 100 bp DNA ladder (Gibco/BRL). Keratinocyte yield determined daily for 5 days as shown in Figure 9B demonstrates that within 24 hours there is a 50% decrease

20 in cell yield in cultures provided with diluent alone but on 30% decreases in cultures provided with NGF. UV irradiated keratinocytes were growth arrested as expected. However, cell yields of keratinocytes maintained in NGF supplemented medium increased by 40% within the 5 days of

25 the experiment, suggesting that NGF is a mitogen for keratinocytes as well as a survival factor. This experiment demonstrates that, similar to melanocytes, NGF is a survival factor for keratinocytes. Furthermore, the experiment suggests that NGF might be a mitogen for

30 keratinocytes as well.

EXAMPLE 11: KERATINOCYTE EXPRESSION IN BCL-2 PROTEIN AFTER NGF DEPRIVATION

To determine if NGF affects BCL-2 protein level in keratinocytes, cells were maintained in Keratinocyte basal medium until 60-80% confluent. Then cells were provided medium lacking BPE to eliminate exogenous NGF. Duplicate
5 cultures were provided 50 ng/ml NGF or diluent alone.

Total keratinocyte proteins were extracted, and BCL-2 levels were determined by Western blot analysis as in Example 6. Figure 10 shows that within 24 hours of NGF depletion decreased BCL-2 level was detected in diluent
10 supplemented keratinocytes as compared to NGF supplemented cells. Similar findings were observed at 48 hours. This experiment demonstrates that NGF contributes, at least in part, to BCL-2 maintenance keratinocytes.

EXAMPLE 12: THE EFFECT OF β-AMYLOID ON NORMAL HUMAN
15 MELANOCYTES

To determine the effect of β-amyloid on normal human melanocytes, cultures were supplemented with increasing concentrations (0.025 - 50 uM) of HPLC-purified β-amyloid fragment corresponding to amino acids 1-40. A peptide
20 containing the first 40 amino acids synthesized in reverse order (40-1) was used as a negative control.

Melanocytes were maintained in serum free Medium 199 (Gibco BRL Gaithersburg, MD) supplemented with epidermal growth factor (10 ng/ml) (Collaborative Research), insulin
25 (10 μg/ml) (Sigma), triiodothyronine (10^{-9} M)(Collaborative Research), transferrin (10 μg/ml) (Sigma), hydrocortisone (1.4 x 10^{-6} M) (Calbiochem), cholera toxin (10^{-9} M) (Calbiochem) and basic fibroblast growth factor (basic FGF) (10 ng/ml) (Collaborative Research). Two days after
30 plating cells were supplemented with increasing concentrations of β-amyloid 1-40 or the control peptide 40-1 (0-50 μM) (Bachem California, Torrance, CA). Cell yields determined three days after addition of β-amyloid show a

dose dependent decrease of cell yield in cultures maintained with the 1-40 peptide. No effect on cell yield was observed in cultures maintained with the control 40-1 peptide.

5   Melanocytes maintained in the presence of 25-30 uM β-amyloid 1-40 showed a 59% ± 17% decrease in cell yield as compared to cell yield before β-amyloid addition that is considered to be 100%. Cell yield of duplicate cultures maintained in the presence of the control 40-1 peptide

10   showed 8% ± 32% increase in cell yield (p<0.02, paired t test).

       Melanocytes maintained in the presence of the 40-1 control peptide have a typical bipolar to polygonal morphology. The majority of the melanocytes maintained in

15   the presence of the 1-40 peptide are rounded and detaching from the dish surface.

       Regression analysis showed significant decrease in cell yield with increasing concentrations of β-amyloid 1-40 (R^2=0.8475, p<0.00001) but no significant effect on cell

20   yield of β-amyloid 40-1 (R^2=0.06, p=0.44). In a total of four experiments, within 3-5 days β-amyloid 1-40 decreased melanocyte yield by >50% (p<0.02; paired t test) while the control 40-1 β-amyloid peptide at the same concentrations had no effect on cell yield.

25   EXAMPLE 13: EFFECT OF β-AMYLOID 1-40 ON MELANOCYTE PLAQUE FORMATION

       Melanocyte cultures, cultured as described above in Example 12, were also evaluated for plaque formation. In some cultures the development of plaque-like structures

30   consisting of increasingly large congregations of dying melanocytes was noted, reminiscent of the senile plaques described in the brains of patients with Alzheimer's disease.

EXAMPLE 14: EFFECT OF β-AMYLOID AND NGF ON MELANOCYTES

In neurons, the protein product of the proto-oncogene Bcl-2 delays the onset of apoptosis triggered by a variety of stimuli, while overexpression of a Bcl-2 associated

5   protein (Bax) accelerates this cell death.

To investigate the mechanism of β-amyloid-mediated melanocyte death, Bax levels in melanocytes treated with 25 uM of 1-40 or 25-35 β-amyloid peptides was examined. Within 4 days of treatment, Bax was induced 3 fold in

10 melanocytes stimulated either with the β-amyloid 1-40 or 25-35 fragments as compared to melanocytes treated with the 40-1 control fragment or an irrelevant HPLC purified protein of similar size.

Melanocytes were maintained as above. Four days after

15 addition of 25 μM of β-amyloid fragments 1-40 40-1 or 25-35; or 25 μM of HPLC-purified bovine corticotropin releasing factor (CRF) (Bachem California) (MW 4.7 kD) as an additional negative control, cells were extracted in RIPA buffer (50 mM Tris-HCl [pH 8.0], 0.15 M NaCl, 0.5%

20 sodium deoxycholate, 1% Triton X-100) in the presence of 1 μg/ml aprotinin and 75 μg/ml phenylmethylsulfonyl fluoride (PMSF), sonicated for 1-3 seconds and centrifuged. 40 μg of protein per lane were separated on 12% SDS/PAGE and blotted onto nitrocellulose paper (overnight, 25V). To

25 verify equal loading a duplicate 13% SDS/PAGE was performed and stained with Coomasie Blue R250 stain. Blots were incubated with anti-Bax antibody (1:1000 dilution) (primary antibody), followed by horseradish peroxidase-conjugated goat anti-rabbit IgG (secondary antibody) (1:500 dilution)

30 (Bio-Rad Laboratories, Hercules, CA). Bound antibodies were detected using enhanced chemiluminescence kit (Amersham Corp.). Autoradiograms were scanned into a Macintosh II is computer using the Ofto TM program (Light source Computer Images, Inc.). Scan analysis was performed

with manual definition of bands using Scan Analysis TM
68000 program (Biosoft, Cambridge, UK).

β-amyloid peptides 1-40 and 25-35 increased Bax level
as compared to the 40-1 peptide (270% and 160%,
5 respectively).

EXAMPLE 15: EFFECT OF NGF ON β-AMYLOID INDUCED CELL DEATH

Nerve growth factor (NGF) has been reported to delay
melanocyte apoptosis by upregulating the levels of Bcl-2
(Zhai, S., et al. Exp. Cell. Res.. It was investigated
10 whether NGF supplementation protects melanocytes from β-
amyloid-induced cell death. Addition of NGF to β-amyloid-
supplemented melanocytes enhanced cell yields within 3-5
days and greatly improved the morphology of surviving cells
in most donors, although degree of protection was variable
15 among donors. Preliminary data suggest that NGF
supplementation decreases the β-amyloid-induced Bax
upregulation and increases Bcl-2 levels in the cells
suggesting that NGF interferes with β-amyloid-mediated
signal transduction.

20 Melanocytes were maintained as above in hormone
supplemented medium lacking hydrocortisone. Cells were
supplemented with 25 μM of β-amyloid 1-40 in the presence
of 50 ng/ml NGS or diluent.

Photographs of representative fields were obtained and
25 the percent of the live cells (spread) was determined 48
hours after addition of β-amyloid and NFG or β-amyloid and
diluent. In the presence of β-amyloid and diluent 77±8.5%
appeared spread as compared to 96±1.4% in cultures
supplemented in β-amyloid and NGF. In cultures not
30 provided β-amyloid and diluent even the cells that were
still spread on the dish surface were vacuolated and
generally did not appear as healthy as cells in NGF
supplemented cultures.

β-amyloid exposed melanocytes in the absence of NGF are dying, while in the presence of NGF the cells appear healthy and spread on the dish surface. At least 400 cells were counted in each condition.

5 EXAMPLE 16: β-AMYLOID BINDS THE p75 NERVE GROWTH FACTOR RECEPTOR

To determine if β-amyloid binds $p75^{NTR}$, ^{125}I 1-40 β-amyloid peptide was added to permanently transfected cultured fibroblasts that overexpress $p75^{NTR}$ ($p75^{NTR}$-NIH 3T3

10 cells) (Dobrowsky, T.T., et al., Science, 265:1596 (1994) in the presence of disuccinimidyl suberate to cause cross linking of closely associated proteins. Cells were then immunoprecipitated with anti $p75^{NTR}$ antibodies or an irrelevant mouse IgG. Autoradiograms revealed a protein

15 band of 75-80 kD in size only in lysates immunoprecipitated with anti $p75^{NTR}$ antibodies. Competition analysis of ^{125}I 1-40 β-amyloid in the presence of increasing concentrations of unlabeled NGF showed that 1-40 β-amyloid could be competed off by NGF. However, residual ^{125}I 1-40 binding

20 suggests that 1-40 β-amyloid may have an additional cell surface receptor, perhaps the recently identified serpin-enzyme complex receptor.

Results indicate that $p75^{NTR}$ is a receptor for β-amyloid, a peptide that is reported to be secreted into the

25 medium of normal cells in high picomolar to low nanomolar concentrations.

$p75^{NTR}$-NIH 3T3 cells were maintain in DMEM supplemented with 10% FBS in the presence of penicillin (45 ng/ml), streptomycin (68 ng/ml), and hygromycin B (17.5 ng/ml).

30 At 80% confluence cells were lifted from the dish with EDTA and incubated in suspension with 5 μCi ^{125}I β-amyloid 1-40 at 4°C for 1 hour in DMEM. After incubation, 1 mM of disuccinimidyl suberate was added for 30 minutes.

Following centrifugation cells were lysed with RIPA buffer (50 mM Tris HCl, pH 8.0, 0.15 M NaCl 0.5% sodium deoxycholate 4, 5 mM $MgCl_2$, 1% Triton x-100, 1 mM phenylmethylsulfonylfluoride [PMSF], and 1 μg/ml

5 aprotinin), sonicated for 1-3 seconds and immunoprecipitated with anti p75^{NTR} antibodies (mouse monoclonal IgG1, Cedarlane Laboratories Ltd., Ontario, Canada) or mouse IgG as control for 16 hours at 40°C in the presence of 15 μl of protein G plus protein A agarose and

10 1M NaCl adjusted to pH 8.0. After several washes with 20 mM Tris HCl, pH 8.0, 1M NaCl, 5mM $MgCl_2$, 0.2% Triton X-100 and 1 mM PMSF, immunoprecipitates were separated over 8% PAGE and subjected to autoradiography. A band of 75-80 kD molecular weight was present only in lysates

15 immunoprecipitated with anti p75^{NTR} antibodies and not lysates immunoprecipitated with irrelevant mouse IgG.

p75^{NTR}-NIH 3T3 cells were incubated for 2 hours at 40°C in binding medium (DMEM, 10 mM hepes, 0.1 mg/ml cytochrome C, 0.01% Tween 80, 1 mg/ml BSA) with ^{125}I β-amyloid 1-40 and

20 increasing concentrations of NGF (0-100 ng/ml). After rinsing in PBS cells were lysed in 1N NaOH and equal amounts of protein from cell lysates were subject to γ counting. A concentration dependent inhibition of ^{125}I β-amyloid binding by NGF was observed with a maximum of 38%

25 inhibition at NGF concentrations of 100 ng/ml and statistically comparable binding at 25 ng/ml.

EXAMPLE 17: KGA-CONTAINING PEPTIDES COMPETITIVELY INHIBIT β-AMYLOID BINDING TO p75^{NTR}

p75^{NTR}-NIH-3T3 cells were incubated in suspension at

30 4°C for 4 hours with 0.5 μCi^{125}I β-amyloid 1-40 and increasing concentrations (0-400 nM) of the cyclic peptide CVGSNKGAIC (SEQ ID NO: 4). Lysates from 1.5 X 10^5 cells were subjected to counting. As shown in Figure 11,

concentration dependent inhibition of $^{125}I\beta$-amyloid 1-40 binding by the cyclic peptide was observed with 50% inhibition was observed at the expected 25 nM cyclic peptide concentration. This experiment demonstrates the

5 cyclic peptide can compete with β-amyloid 1-40 for binding to the p75^{NTR} receptor.

EXAMPLE 18: EFFECT OF KGA-CONTAINING PEPTIDES ON CELL SURVIVAL

      p75^{NTR}-NIH 3T3 cells were maintained in DME

10 supplemented with 10% calf serum until 80% confluent. The cells were washed and incubated in serum free DME containing transferrin (5 μg/ml) and insulin (5 μg/ml). Triplicate dishes were supplemented with diluent or preaggregated β-amyloid 1-40 (250nM) as positive and

15 negative controls, respectively. Figure 12A shows the results from triplicate dishes supplemented with cyclic decapeptide CVGSNKGAIC (250nM, SEQ ID NO:4) alone or together with preaggregated β-amyloid 1-40 (250nM). Figure 12B shows the results from triplicate dishes supplemented

20 with cyclic decapeptide CATDIKGAEC (250nM, SEQ ID NO:9) alone or together with preaggregated β-amyloid 1-40 (250nM). Figure 12C shows the results from triplicate dishes supplemented with cyclic hexapeptide CKGAIC (250nM, SEQ ID NO:10) alone or together with preaggregated β-

25 amyloid 1-40(250nM). After 72 hours, cells were rinsed in PBS and cultures were incubated in 0.25% trypsin at 37°C. Cell yields were determine using a particle counter. Cell yields in cultures supplemented with each cyclic peptide and β-amyloid were significantly higher than cell yields of

30 cultures supplemented with β-amyloid alone (p<0.01 CVGSNKGAIC, p<0.0002 CATDIKGAEC, p<0.002 CKGAIC; non-paired t test comparing the effect of β-amyloid and peptide to β-

amyloid alone. Test was performed separately for each group).

Equivalents

Those skilled in the art will recognize, or be able to
5 ascertain using no more than routine experimentation, many
equivalents to the specific embodiments of the invention
described herein. Such equivalents are intended to be
encompassed by the following claims.